Confidential MicroBioTest Protocol

Testing Pre-Saturated or Impregnated Towelettes for Tuberculocidal Effectiveness

Testing Facility
MicroBioTest
Division of Microbac Laboratories, Inc.
105 Carpenter Drive
Sterling, VA 20164

Prepared for
METREX RESEARCH, LLC
1515 S Manchester Avenue
Anaheim, CA 92802

March 2, 2015

MicroBioTest, Division of Microbac Laboratories, Inc.
105 Carpenter Drive|Sterling, VA20164| 703.925.0100 p |703.925.9366 f| www.microbac.com

OBJECTIVE:

This test is designed to substantiate tuberculocidal effectiveness for impregnated or pre-saturated towelettes, single or multiple uses, to be registered with the Environmental Protection Agency and Health Canada. The test incorporates appropriate aspects AOAC method 965.12 Tuberculocidal Activity of Disinfectants (2012). The test evaluates the effectiveness of products as disinfectants for contaminated surfaces. The test follows the "Germicidal Spray Products as Disinfectants" test as described in the Official Methods of Analysis, Eighteenth Edition, 2012, AOAC. This test also meets the EPA OCSPP 810.2000 and 810.2200 Product Performance Test Guidelines as applicable and follows the US. EPA OPP Microbiology Laboratory, SOP for Disinfectant Towelette Test against Mycobacterium bovis (BCG), SOP Number: MB-23-02, Date Revised: 03-05-13 where appropriate.

TESTING CONDITIONS:

A total of ten carriers per lot will be evaluated using two lots of a single test agent. The carriers, inoculated with *Mycobacterium bovis* (BCG), will be wiped following the procedure described in the protocol and held for the exposure time and at the temperature specified by the sponsor. The carriers will be cultured, incubated and observed for visible growth.

MATERIALS:

A. Test agents supplied by the sponsor: see last page. As per CFR 40.160.105:

- The identity, strength, purity, and composition, or other characteristics which will appropriately define the test, control, or reference agent shall be determined for each lot and shall be documented by the sponsor before its use in a study. Methods of synthesis, fabrication, or derivation of the test, control, or reference agent shall be documented and retained by the sponsor.

- When relevant to the conduct of the study the solubility of each test, control, or reference agent shall be determined by the sponsor before the experimental start date. The stability of the test, control, or reference agent shall be determined by the sponsor before the experimental start date or concomitantly according to written standard operating procedures, which provide for periodic analysis of each lot.

JT

- The test agent will be tested as supplied by the sponsor unless directed otherwise. All operations performed on the test agent such as dilution or specialized storage conditions must be specified by the sponsor before initiation of testing.
- The sponsor assures MicroBioTest, a Division of Microbac Laboratories, Inc. (MicroBioTest) testing facility management that the test agent has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

- MicroBioTest will retain all unused test agents for a period of at least one year after completion of the test, and then discard them in a manner that meets the approval of the safety officer.

B. Materials supplied by MicroBioTest including but not limited to:

1. Challenge organism, required by AOAC:
   Mycobacterium bovis (BCG). OrganonTeknika, Corp.

2. Media and reagents:
   a. Middlebrook 7H11 agar (7H11).
   b. Kirchners medium (KM).
   c. Middlebrook 7H9 broth (7H9).
   d. Modified Proskauer-Beck Medium (MPB).
   e. Phosphate Buffered Dilution Water (PBDW).
   f. 0.85% NaCl containing 0.1% Polysorbate 80 (SS+).
   g. Sterile Saline Solution.
   h. Heat-inactivated Fetal Bovine Serum (FBS), if required.
   i. Neutralizer: MPB containing 7% Polysorbate 80 and 1% Lecithin

3. Laboratory equipment and supplies.

4. Laboratory equipment and supplies, including glass microscope slides (1" x 3" with a 1" x 1" surface for contamination and treatment)

TEST SYSTEM IDENTIFICATION:

All test and control tube racks will be labeled with microorganism, test agent (if applicable) and project number prior to initiation of the study and during incubation. Petri dishes will be labeled with microorganism prior to initiation of the study and microorganism and project number during incubation.

JT

EXPERIMENTAL DESIGN:

A. Preparation of inoculum:

From stock culture, tubes containing 20 mL of MPB will be inoculated by transferring one or two 1 µL loopfuls from 7H11 slants and incubated in a slanted position at 36±1C while remaining quiescent for 21±2 days.

Using a transfer loop, sufficient growth will be transferred from the surface of the 20 mL culture into a sterile tissue grinder. One mL of SS+ will be added and the culture will be macerated to break up large clumps. Nine mL of MPB will be added to the homogenized culture. The homogenized suspension will be transferred to a sterile tube and the culture will be allowed to settle for 10-15 minutes. The upper portion of the culture will be removed and transferred to a sterile flask, leaving behind any debris or clumps.

Using a spectrophotometer, the culture suspension will be standardized using MPB to achieve 20.0%±1% transmittance (T) at 650 nm.

If requested by the sponsor, Heat-inactivated FBS will be added to the culture to achieve 5% organic load.

B. Preparation of carriers:

The new carriers will be visually screened and discarded if visibly damaged (scratched, chipped or nicked). The carriers will be rinsed with 95% ethanol followed by a rinse with deionized water to remove oil and film on the slides. The carriers will be sterilized by placing in evaporating dishes matted with two pieces of filter paper, heating them in a hot air oven for two hours at 180C, cooling and storing them at room temperature until use.

C. Carrier inoculation:

Using a positive displacement pipette, a 0.01 mL (10 µL) aliquot of the culture will be transferred onto a one-square inch area on the sterile carriers (in Petri dishes) and immediately spread uniformly over the entire area with a sterile loop. Each dish will be covered promptly and the operation will be repeated for the rest of the carriers. Carriers will be dried for 30±2 minutes at 36±1C. Inoculated carriers will be used for testing within two hours of drying.

Note: The temperature and humidity level of the incubator during the drying of carriers will be monitored and reported.

JT

D. Test material preparation:

The test agent will be used as received by the sponsor of the study. Each canister of test agent will be allowed to equilibrate to room temperature for a minimum of two hours. The canisters or containing the towelettes will be inverted or mixed to re-saturate the towelettes.

E. Test:

For each lot of test material, ten carriers will be treated using the contact time stipulated by the sponsor. When using towelettes from a new canister, the lid will be removed and the center of the roll pulled out and inserted into the lid opening. The lid will be replaced and the towelette pulled through the lid opening, making it ready for use in testing.

At least five wipes will be removed from the canister and discarded before use in testing.

Initially the towelette will be folded lengthwise twice and then folded five times inward beginning from the far end. Then the outside edges will be pulled upward to form a "U" shape and grasped preferably on one side with the thumb and on the other side with the index and middle finger. The folded towelette will be rotated 90°.

Ten (10) carriers will be treated using the following procedures requested by the sponsor. Each carrier will be wiped back and forth three times lengthwise with the towelette for a total of six passes across the inoculum. Wiping will be done within ± 5 seconds. The lid of the Petri dish will be closed. The wiped carriers will be maintained in a horizontal position.

The used end of the wipe will be flipped upward towards self, reoriented appropriately and then used to wipe the next carrier. The next three carriers will be wiped in a similar fashion - the used portion will be folded up-and-over each time.

JT

Once five carriers have been wiped, the towelette will be unraveled. The second lengthwise fold will be unfolded and refolded in the opposite direction. The towelette will be refolded five times as before and the above procedure for wiping the first five carriers will be repeated for wiping the last five carriers.

Then each carrier will be wiped with a second towelette in the same manner as the first. The contact time will be initiated once the carrier is wiped with the second towelette.

After the contact period, the excess liquid will be allowed to drain from the carrier without touching the Petri dish or filter paper. Sequentially, the carriers will be transferred to tubes containing 20 mL of the Neutralizer using sterile forceps within the ±5 sec (or ±3 sec) time limit and shaken thoroughly. For products with ≤1 minute contact time, the transfer will be made within ±3 seconds. The slide could touch both the interior sides of the Petri dish and the neutralizer tube during the transfer, but care will be taken to avoid this contact as much as possible.

Each tube containing carrier in neutralizer will be thoroughly shaken and the carrier will be transferred to a tube containing 20 mL of MPB broth within 5-10 minutes. Sterilize forceps will be used for each carrier transfer. From each tube of neutralizer, two mL will be subcultured to a tube containing 20 mL 7H9 broth and 2 mL will be subcultured to a tube containing 20 mL Kirchner's medium.
Each subculture tube will be shaken thoroughly and the sequence will be repeated for all carriers within 30± 5 minutes.

F. Controls:

1. Viability controls:

One contaminated carrier each will be added to tubes containing 20 mL MPB, 20 mL 7H9, and 20 mL KM. The tubes will be incubated with the test in order to confirm the test organism viability.

2. Neutralizer effectiveness:

The standardized culture will be serially diluted 10-fold with MPB out to the 10^{-6} (e.g., dilution tubes 10^{-3} through 10^{-6}). 100ul aliquots of dilutions 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} will be added per tube to demonstrate the recovery of a low level (<100 CFU/tube) of the test organism in the subculture media as described below,

A single carrier per test culture dilution (e.g., dilution tubes 10^{-3} through 10^{-6}) will be exposed to the test material and processed in the same manner as the test carriers. To each tube of 20 mL 7H9, 20 mL KM and 20 mL MPB media, fewer than 100 CFU will be added to each tube. All tubes will be incubated alongside the test. When inoculated with ≤100CFU/tube, all treatment tubes must demonstrate growth to have a valid neutralization effectiveness test.

A single sterile carrier per test culture dilution (e.g., dilution tubes 10^{-3} through 10^{-6}) will also be evaluated by omitting test material treatment followed by processing in the same manner as the test carriers. To each tube of 20 mL 7H9, 20 mL KM and 20 mL MPB media, fewer than 100 CFU will be added to each tube. All tubes will be incubated alongside the test. When inoculated with 5-100CFU/tube, the MPB tube with the carrier must demonstrate growth to have a valid neutralization effectiveness test.

Untreated tubes of each media per test culture dilution will also be inoculated with 100CFU for comparison. All tubes will be incubated alongside the test. The untreated carrier tubes should exhibit growth for all media. When inoculated with 5-100CFU/tube, all media tubes must demonstrate growth to have a valid neutralization effectiveness test.

The concentration of the bacterial suspension inoculated into these tubes will be confirmed by plating 100 µL aliquots of appropriate dilutions in duplicate on 7H11 using spread plating. The plates will be incubated for 17 – 21 days at 36±1C.

JT

3. Sterility controls:

One sterile, uninoculated carrier will be placed into a tube of MPB broth. In addition, 1 tube of each subculture medium with 2 mL sterile neutralizer will be incubated for quality control purposes. Each tube will be shaken thoroughly and all the tubes will be incubated with the efficacy test. Duplicate 7H11 agar plates will also be incubated with the test.

4. Carrier counts:

Three inoculated dried carriers will be randomly selected for evaluation. One carrier will be assayed immediately prior to conducting the efficacy test and two carriers following the test. Each of three carriers will be placed in independent sterile 50 mL polypropylene conical tubes containing 20 mL of MPB and subjected to vortex for 15 seconds.

After vortex mixing for 15 seconds, 10-fold serial dilutions will be conducted in PBDW. 100 µL aliquots of appropriate dilutions will be plated in duplicate on M7H11 using spread plating. Dilutions 10^{-1} through 10^{-3} should produce plates with CFU in the appropriate range. Plates must be dry prior to incubation. All dilutions and plating will be performed within 2 hours of vortexing. The plates will be incubated for 17-21 days at 36±1C.

The $\log_{10}$ density (LD) for each carrier will be determined based on the following:

- Dilutions yielding counts up to 300 CFU will be used.
- Plate counts of 0 will be included in the calculations.
- The CFU/mL (of broth) will be calculated:

$$\text{CFU/mL} = \frac{(\text{avg. CFU for } 10^{-x}) + (\text{avg. CFU for } 10^{-y}) + (\text{avg. CFU for } 10^{-z})}{10^{-x} + 10^{-y} + 10^{-z}}$$

- The CFU/carrier will be calculated by multiplying the CFU/mL by the volume of broth into which the bacteria were harvested from the carrier by vortexing (20 mL).
- The LD for each carrier will be calculated by taking the Log_{10} of the density (per carrier).
- The mean LD across carriers is the mean Test LD for the test. The mean Test LD must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 6.0 log CFU/carrier (corresponding to a geometric mean density of 1.0×10^6).

JT

5. Performance assessment of Media:

The standardized culture will be serially diluted 10-fold with MPB out to the 10^{-6}. Verify CFU/tube by spread plating in duplicate on 7H11. One of the dilutions plated must demonstrate 5-100 CFU/0.1mL aliquot.

For solid media, 0.1 mL aliquots from the 10^{-3} to the 10^{-6} dilutions will be spread plated on 7H11 agar plates in duplicate. The plates will be incubated for 17-21 days at 36±1C. Media of sufficient quality is achieved when growth is demonstrated following inoculation of 30-300 CFU/plate.

For liquid media, each tube of liquid medium (MPB, 7H9 and KM) will be inoculated with 0.1 mL aliquots from the 10^{-3} to the 10^{-6} dilutions in duplicate. Tubes will be incubated for 60 days at 36±1C. Media of sufficient quality is achieved when growth is demonstrated in at least one of two tubes following inoculation of 5-100 CFU/tube. All tubes receiving a higher level of inoculum should demonstrate growth.

G. Incubation:

All test tubes used for secondary transfers (MPB, 7H9, and KM) and all control tubes will be incubated at 36±1C. Observations for growth or no growth will be made on days 21, 45, 60 and 90 of incubation. All plates will be incubated for 17-21 days at 36±1C, the colonies will be counted and the average CFU calculated.

H. Confirmation of challenge microorganism:

On the day of the final reading, acid-fast stains will be performed for all test culture tubes demonstrating visible growth and two viability control tubes in order to verify the presence of the challenge microorganism. In addition, the culture morphology will be observed.

PRODUCT EVALUATION CRITERIA:

The test agent meets effectiveness requirements if no visible growth occurs in any replicate tube, for any of the subculture broths.

TEST ACCEPTANCE CRITERIA:

The test will be acceptable if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance. There are no proposed statistical methods for this test.

- The mean Test LD for the carrier counts must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 6.0 log CFU/carrier (corresponding to a geometric mean density of 1.0×10^5).
- Test LD that are below 4.0 log CFU/carrier or above 6.0 log CFU/carrier invalidates the test.
- Following inoculation of ≤100 CFU per tube/plate, growth must be observed for the media evaluated in the performance assessment of Media section.
- The sterility controls must show no growth.
- The viability controls must show growth in all media.
- The neutralization confirmation tubes must show growth following inoculation with ≤100 CFU per tube to confirm effective neutralization.

DATA PRESENTATION:

The final report will include the following information:

- The number of positive carriers.
- The average colony-forming units per carrier.
- The results of all controls.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned before initiation of the test. Resumes for technical personnel are maintained and are available on request. This study will be conducted at MicroBioTest, 105 Carpenter Drive, Sterling, VA 20164.

JT

REPORT FORMAT:

The report will contain all items required by 40 FR Part 160.185 and EPA 810.2200 and be in compliance with EPA PR Notice 2011-3 (replaced PRN 86-5). MicroBioTest employs a standard report format for each test design. Each final report will provide at least the following information:

- Sponsor identification
- Test agent identification
- Type of assay and project number
- Study start and end time (clock time)
- Interpretation of results and conclusions
- Test results presented in tabular form
- Methods and evaluation criteria, if applicable
- Dates of study initiation and completion (GLP studies only)
- Signed Quality Assurance and Compliance Statements (GLP studies only)
- Certificate of Analysis (for GLP studies only; to be provided by the Sponsor)
- Draft report will be provided to the Sponsor for review prior to finalization.

CONFIDENTIALITY:

All data generated at MicroBioTest are held in strictest confidence and are available only to the sponsor and the sponsor designated authorities (if applicable). In turn, no reference to MicroBioTest's promotion of the evaluated test articles may be made public by the sponsor.

REGULATORY COMPLIANCE AND QUALITY ASSURANCE (applicable to GLP studies only)

This study will be performed in compliance with the US Environmental Protection Agency's Good Laboratory Practices regulations, 40 CFR 160. Note: information on the identity, strength, purity, stability, uniformity, and dose solution analysis of the test agent resides with the sponsor of the study unless otherwise stated.

The Quality Assurance Unit of MicroBioTest will inspect the conduct of the study for GLP compliance. The dates of the inspections and the dates that findings are reported to the study management and study director will be included in the final report.

RECORDS TO BE MAINTAINED:

All raw data, protocol, protocol modifications, test agent records, final report, and correspondence between MicroBioTest and the sponsor will be stored in the archives at MicroBioTest, 105 Carpenter Drive, Sterling, Virginia 20164 or in a controlled facility off site.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test agent; challenge microorganism used; media and reagent identification; and the type of neutralizers employed in the test will be addressed in a project sheet issued separately for each study. The date the study director signs the protocol will be the initiation date. All project sheets will be forwarded to the study sponsor.

MISCELLANEOUS INFORMATION: The following information is to be completed by sponsor before initiation of study:

A. Name & address: METREX RESEARCH, LLC
1515 S Manchester Avenue
Anaheim, CA 92802

B. Test agent information:

Test Agent Name	CaviWipes	
Active Ingredient(s)	Hyamine 1622 and Isopropyl Alcohol	
Lot No.	Lot 1	Lot 2
	15-2113	15-3117
	testing will be performed at the LCL: ☒ yes ☐ no	
Manufacture Date	4/23/2015	4/27/2015

C. Test conditions:

Contact Time	2 minutes 45 seconds minutes
Contact Temperature	☒ Ambient room temperature (20±1C) ☐ Other:_____
Dilution	Not applicable/ready to use wipes
Diluent	Not applicable/ready to use wipes
Neutralizer	Per sponsor: MPB containing 7% Polysorbate 80 and 1% Lecithin
Soil Load	None

D. Precautions/storage – MSDS or certificate of analysis provided: ☒ yes ☐ no

REPORT HANDLING: The sponsor intends to submit this information to:

☒ US EPA ☐ Health Canada ☐ R&D: Other Internal Purpose

STUDY CONDUCT: ☒ GLP ☐ non-GLP

PROTOCOL APPROVAL:

Sponsor Signature: _____ Date: 4/30/2015

Joe Tulpinski, METREX RESEARCH, LLC

Study Director Signature: _____ Date: 5/13/15

Travis R. Farley